

Coenzyme Q Biosynthesis: Coq6 Is Required for the C5-Hydroxylation Reaction and Substrate Analogs Rescue Coq6 Deficiency

Mohammad Ozeir,^{1,2,3} Ulrich Mühlenhoff,⁴ Holger Webert,⁴ Roland Lill,⁴ Marc Fontecave,^{1,2,3,5} and Fabien Pierrel^{1,2,3,*}¹CEA, iRTSV, Laboratoire Chimie et Biologie des Métaux²CNRS, UMR5249

F-38054 Grenoble, France

³Université Joseph Fourier-Grenoble I, UMR5249, F-38041 Grenoble, France⁴Institut für Zytobiologie und Zytopathologie, Philipps-Universität Marburg, 35032 Marburg, Germany⁵Collège de France, 75005 Paris, France*Correspondence: fabien.pierrel@cea.fr

DOI 10.1016/j.chembiol.2011.07.008

SUMMARY

Coenzyme Q (Q), an essential component of eukaryotic cells, is synthesized by several enzymes from the precursor 4-hydroxybenzoic acid. Mutations in six of the Q biosynthesis genes cause diseases that can sometimes be ameliorated by oral Q supplementation. We establish here that Coq6, a predicted flavin-dependent monooxygenase, is involved exclusively in the C5-hydroxylation reaction. In an unusual way, the ferredoxin Yah1 and the ferredoxin reductase Arh1 may be the *in vivo* source of electrons for Coq6. We also show that hydroxylated analogs of 4-hydroxybenzoic acid, such as vanillic acid or 3,4-dihydroxybenzoic acid, restore Q biosynthesis and respiration in a *Saccharomyces cerevisiae* *coq6* mutant. Our results demonstrate that appropriate analogs of 4-hydroxybenzoic acid can bypass a deficient Q biosynthetic enzyme and might be considered for the treatment of some primary Q deficiencies.

INTRODUCTION

Coenzyme Q or ubiquinone (Q) is a redox active lipid present in most organisms and in all tissues of multicellular eukaryotes where it shuttles electrons from complex I and II to complex III of the mitochondrial respiratory chain and acts as an important lipid-soluble antioxidant. Moreover, Q participates in the control of the mitochondrial membrane transition pore and functions with uncoupling proteins in the mitochondrial inner membrane (Bentinger et al., 2010). Q contains a polyprenyl tail with six isopentenyl units in *Saccharomyces cerevisiae* (Q₆) and 10 in human (Q₁₀) (Kawamukai, 2009) (Figure 1). In *S. cerevisiae*, the biosynthesis of Q is accomplished by multiple conserved mitochondrial matrix enzymes (termed Coq1–Coq9) some of which are assembled in a large Q biosynthetic complex associated with the inner membrane (Tran and Clarke, 2007). Mutations affecting five genes involved in Q biosynthesis have been described and result

in primary Q deficiencies that cause clinically heterogeneous diseases (Quinzii and Hirano, 2010). Oral Q₁₀ supplementation of patients yields significant improvement in some cases (Quinzii and Hirano, 2010). Very recently, mutations in a sixth gene, COQ6, were shown to cause nephrotic syndrome with sensorineural deafness (Heeringa et al., 2011).

The initial stage of Q biosynthesis involves Coq2. In *S. cerevisiae*, Coq2 prenylates 4-hydroxybenzoic acid (4-HB) or the newly identified precursor para-aminobenzoic acid (pABA) to yield 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB), respectively (Figure 1) (Marbois et al., 2010; Pierrel et al., 2010). The C4-amine originating from pABA is subsequently converted into an hydroxyl at an unidentified step of Q₆ biosynthesis (Figure 1). *S. cerevisiae* cells depleted for either the mitochondrial ferredoxin Yah1 or the mitochondrial ferredoxin reductase Arh1 synthesize almost no Q₆ and accumulate 3-hexaprenyl-4-aminophenol (4-AP) upon culturing in the presence of pABA or 3-hexaprenyl-4-hydroxyphenol (4-HP) in the presence of 4-HB (Figure 1) (Pierrel et al., 2010). This result established that Yah1 and Arh1 are absolutely required for the C5-hydroxylation step. Arh1 and Yah1 form a well established electron transfer complex necessary in *S. cerevisiae* for iron sulfur cluster (ISC) and heme A biosynthesis (Barros et al., 2002; Lange et al., 2000; Li et al., 2001). Yah1 has two human homologs, Fdx1 and Fdx2. Fdx1 functions in steroid biogenesis by transferring electrons to mitochondrial cytochrome P450 enzymes whereas Fdx2 transfers electrons for the biogenesis of heme A and ISC (Sheftel et al., 2010).

Coq6 is required for Q₆ biosynthesis in *S. cerevisiae* (Gin et al., 2003) and, based on its amino acid sequence, has been predicted to belong to the class A flavoprotein monooxygenase family suggesting that it contains a FAD cofactor (van Berkel et al., 2006). Coq6 has been postulated to function in the C1- and/or the C5-hydroxylation reactions (Gin et al., 2003) (Figure 1). This ambiguity has not been resolved partly because a *S. cerevisiae* mutant lacking the entire COQ6 gene (Δ coq6) accumulates only the early Q₆ biosynthetic intermediate, HHB when grown in the presence of 4-HB (Gin et al., 2003). In fact, the Δ coq3–9 mutants all accumulate HHB because most Coq polypeptides (excluding Coq1, Coq2, Coq5, and Coq8) are interdependent

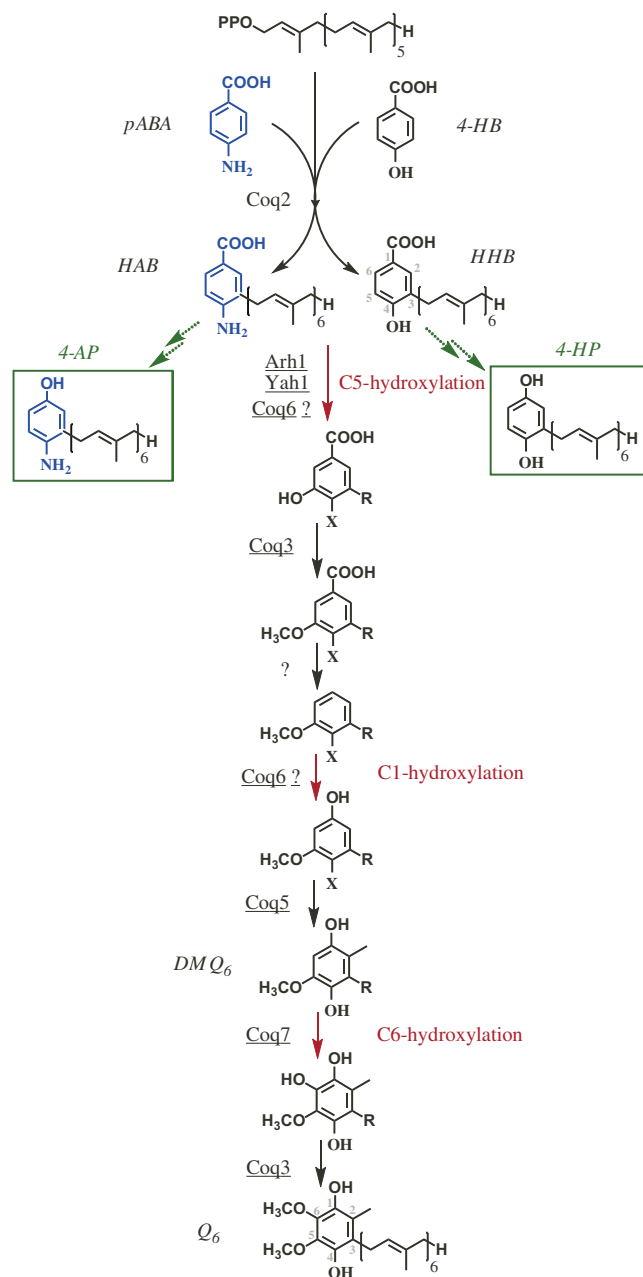


Figure 1. Current Model of the *S. cerevisiae* Coenzyme Q Biosynthetic Pathway

The names of proteins (underlined) and intermediates (italicized) relevant to this study are for *S. cerevisiae*. The numbering of the aromatic carbon atoms used throughout this study is shown on HHB and Q₆. Prenylation of 4-hydroxybenzoic acid (4-HB) or para-aminobenzoic acid (pABA) by Coq2 yields 3-hexaprenyl-4-hydroxybenzoic acid (HHB) or 3-hexaprenyl-4-aminobenzoic acid (HAB). In subsequent reactions, R stands for the hexaprenyl tail and X designates NH₂ or OH. NH₂ is converted into OH prior to demethoxyubiquinone (DMQ₆) formation (Marbois et al., 2010; Pierrel et al., 2010). The three hydroxylation reactions necessary to yield Q₆ are shown in red and the potential implication of Coq6 in the C5- and/or the C1-hydroxylation reactions is indicated (Coq6?). In cells depleted for Yah1 or Arh1, the C5-hydroxylation is deficient and results in synthesis of 3-hexaprenyl-4-aminophenol (4-AP) from HAB and of 3-hexaprenyl-4-hydroxyphenol (4-HP) from HHB (green dashed arrows and green boxes) (Pierrel et al., 2010).

for their stability within the Q biosynthetic complex (Hsieh et al., 2007). Indeed, the absence of a single Coq polypeptide causes the degradation of other Coq proteins (Tran and Clarke, 2007), thus preventing the biosynthesis of Q₆ intermediates diagnostic of the altered step in Δ coq mutants. Consequently, it has been difficult to elucidate the precise function of Coq4, Coq6, and Coq9. Coq8, a predicted protein kinase, is essential for Q₆ biosynthesis and participates in the phosphorylation of Coq3, Coq5, and Coq7 (Tauche et al., 2008; Xie et al., 2011). Overexpression of Coq8 has been shown to restore the levels of Coq3 and Coq4 in most Δ coq mutants (Zampol et al., 2010). In addition, overexpression of Coq8 in a Δ coq7 strain causes the accumulation of demethoxyquinone (DMQ₆) (Padilla et al., 2009), the substrate of the C6-hydroxylase, Coq7 (Stenmark et al., 2001) (Figure 1). This result implies that overexpression of Coq8 in the Δ coq7 strain prevents the degradation of the Coq polypeptides involved in Q₆ biosynthetic steps upstream of Coq7. DMQ₆ also forms in a Δ coq7 strain expressing the inactive allele COQ7-E233K even in the absence of Coq8 overexpression (Padilla et al., 2004). It therefore seems that single mutations in a given Coq protein have less impact on the integrity of the Q₆ biosynthetic complex than null mutations.

In the current study, we sought to define which hydroxylation step of Q₆ biosynthesis is dependent on Coq6. We show that a Δ coq6 strain expressing inactive COQ6 alleles or overexpressing Coq8 accumulates products of the Q₆ biosynthetic pathway that establish that the monooxygenase Coq6 is specifically required for the C5-hydroxylation reaction. The functional combination of Yah1, Arh1, and Coq6 in the C5-hydroxylation reaction is discussed. In addition, we demonstrate that hydroxylated forms of 4-HB like vanillic acid or 3,4-dihydroxybenzoic acid are able to restore Q₆ biosynthesis and respiration in a *S. cerevisiae* strain deficient for Coq6. This represents the first indication that the use of 4-HB analogs might be considered as a strategy to bypass defective steps in the Q biosynthetic pathway.

RESULTS

Coq6 Is Required for the C5- But Not the C1-Hydroxylation Reaction of Q Biosynthesis

Regular synthetic yeast growth medium contains pABA that is a precursor of Q₆ (Marbois et al., 2010; Pierrel et al., 2010). In order to control the nature of the precursors employed for Q₆ biosynthesis, we used a synthetic medium without pABA for all our experiments. Electrochemical detection (ECD) of cell lipid extracts separated by HPLC revealed that addition of pABA or 4-HB to the growth medium increased the Q₆ and DMQ₆ content of *S. cerevisiae* (see Figure S1A available online) consistent with our previous conclusion that endogenous 4-HB is limiting for Q₆ biosynthesis (Pierrel et al., 2010). Overexpression of Coq8 in the Δ coq6 mutant strain led to an accumulation of 4-AP or 4-HP in cells cultured in the presence of pABA or 4-HB, respectively, whereas no products were formed in the absence of Coq8 overexpression (Figure 2A). Identities of 4-AP and 4-HP were established previously (Pierrel et al., 2010) and here confirmed by their retention time in HPLC chromatograms, their ultraviolet-visible spectra (Figure S1B) and the expected ion transitions (*m/z*: 518.3/122 for 4-AP and 519.3/123 for 4-HP) in mass spectrometry coupled to HPLC (data not shown). Without pABA or

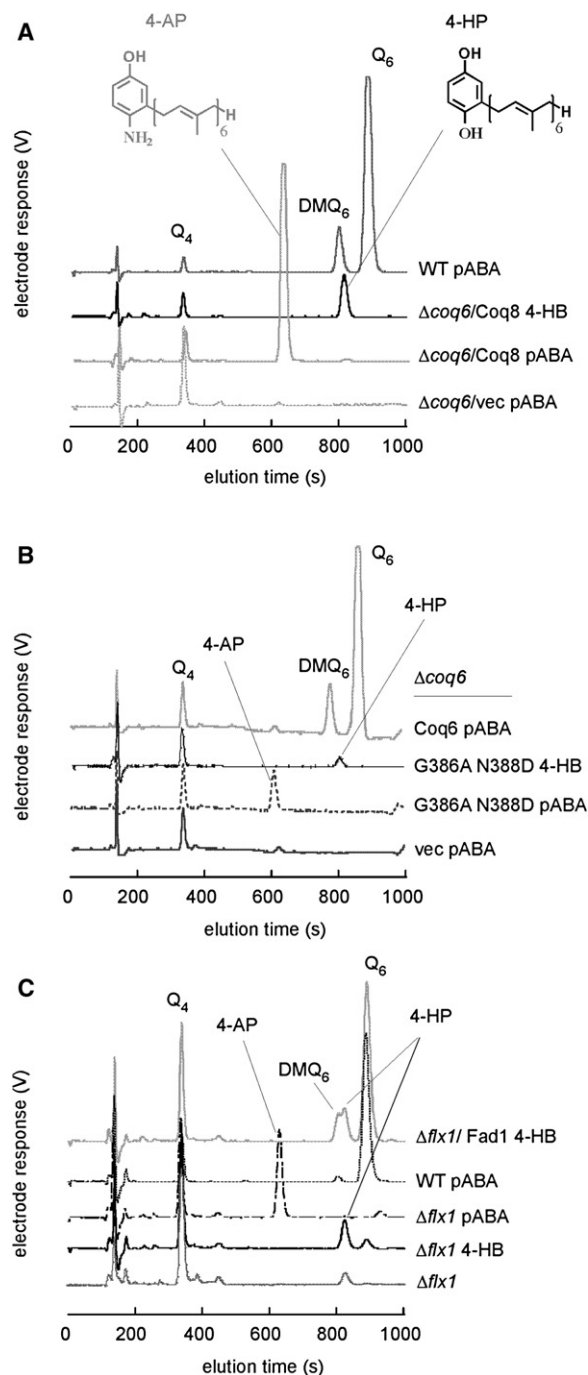


Figure 2. Coq6 Is Required for the C5 but Not for the C1-Hydroxylation of Q₆ Biosynthesis

(A) WT cells or $\Delta coq6$ cells transformed with an empty vector (vec) or with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing or not 100 μ M 4-hydroxybenzoic acid (4-HB) or 100 μ M pABA. Lipid extracts of 1 mg of cells (WT), 2 mg of cells (/Coq8), or 8 mg of cells (/vec) were analyzed by HPLC-ECD. Chemical structures of 3-hexaprenyl-4-aminophenol (4-AP, eluting at 610 s) and of 3-hexaprenyl-4-hydroxyphenol (4-HP, eluting at 810 s) are displayed. The peaks corresponding to coenzyme Q₆ (Q₆), demethoxyquinone (DMQ₆), and to the Q₄ standard are indicated. (B) $\Delta coq6$ cells transformed with an empty vector (vec) or centromeric vectors coding either for Coq6 or Coq6-G386A-N388D were grown in glucose

4-HB supplementation of the growth medium, the $\Delta coq6$ strain overexpressing Coq8 accumulated 4-HP suggesting that endogenous 4-HB enters the Q biosynthetic pathway preferentially over endogenous pABA (Figure S1C). We also mutated residues in Coq6 (G202, G386, N388) selected on the basis of their conservation in other flavoprotein monooxygenases (Figure S2) in which they are implicated in the binding of NADPH and FAD, as shown in the case of the parahydroxybenzoate hydroxylase PcbA (Eppink et al., 1997; Palfey et al., 1994). As expected, a vector coding for Coq6 restored Q₆ biosynthesis in the $\Delta coq6$ strain whereas G202A and G386A-N388D mutant alleles of COQ6 were inactive as proven by the absence of Q₆ and DMQ₆ (Figure 2B; Figure S1C). However, these mutants caused an accumulation of 4-AP in $\Delta coq6$ cells cultured in the presence of pABA, and of 4-HP upon growth in the presence of 4-HB (Figure 2B). Together, these results show that a $\Delta coq6$ strain either expressing a Coq6 protein inactivated by point mutations or overexpressing Coq8 accumulates 4-AP and 4-HP. This finding demonstrates that the C1- but not the C5-hydroxylation reaction is still catalyzed in the absence of Coq6 activity (Figure 1). Because Coq6 is a predicted FAD-dependent monooxygenase (Gin et al., 2003; van Berkel et al., 2006), we checked whether the C5-hydroxylation is dependent on the availability of FAD in mitochondria. Deletion of the gene *FLX1* that codes for a mitochondrial FAD transporter results in decreased activity of FAD-dependent mitochondrial enzymes (Bafunno et al., 2004; Tzagoloff et al., 1996). The $\Delta flx1$ strain showed markedly diminished levels of Q₆ and accumulated 4-AP and 4-HP when cultured in the presence of pABA and 4-HB respectively (Figure 2C). Furthermore, we observed that overexpression of Fad1, the flavin adenine dinucleotide synthetase, restored Q₆ biosynthesis in the $\Delta flx1$ strain, however without completely abolishing the formation of 4-HP (Figure 2C). This result is in agreement with the overexpression of Fad1 being able to partially complement the mitochondrial FAD deficiency of a *flx1* mutant strain (Wu et al., 1995). Collectively, our results show that the C5-hydroxylation depends on mitochondrial FAD and that Coq6, a predicted FAD-dependent monooxygenase, is essential for the C5- but not for the C1-hydroxylation reaction of Q₆ biosynthesis.

Vanillic Acid and 3,4-Dihydroxybenzoic Acid Restore Q₆ Biosynthesis in Strains Lacking Coq6 Activity

We tested whether hydroxylated forms of 4-HB like 3,4-dihydroxybenzoic acid (3,4-diHB) or vanillic acid (VA) (Figure S3A) may enter the Q₆ biosynthetic pathway and therefore bypass the C5-hydroxylation step that is deficient in *coq6* mutants. Addition of 3,4-diHB or VA to the growth medium restored Q₆ biosynthesis in a dose-dependent manner in the $\Delta coq6$ strain overexpressing Coq8 (Figure 3A; Figure S3B) or expressing the inactive Coq6 mutant G386A-N388D (Figure S3C). The $\Delta coq6$

synthetic medium without pABA containing 100 μ M pABA or 100 μ M 4-HB. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(C) WT and $\Delta flx1$ cells transformed or not with an episomal vector coding for Fad1 were grown in glucose synthetic medium without pABA supplemented or not with pABA or 4-HB at 100 μ M. Lipid extracts of 4 mg of cells ($\Delta flx1$) and 1 mg of WT were analyzed by HPLC-ECD.

See also Figures S1 and S2.

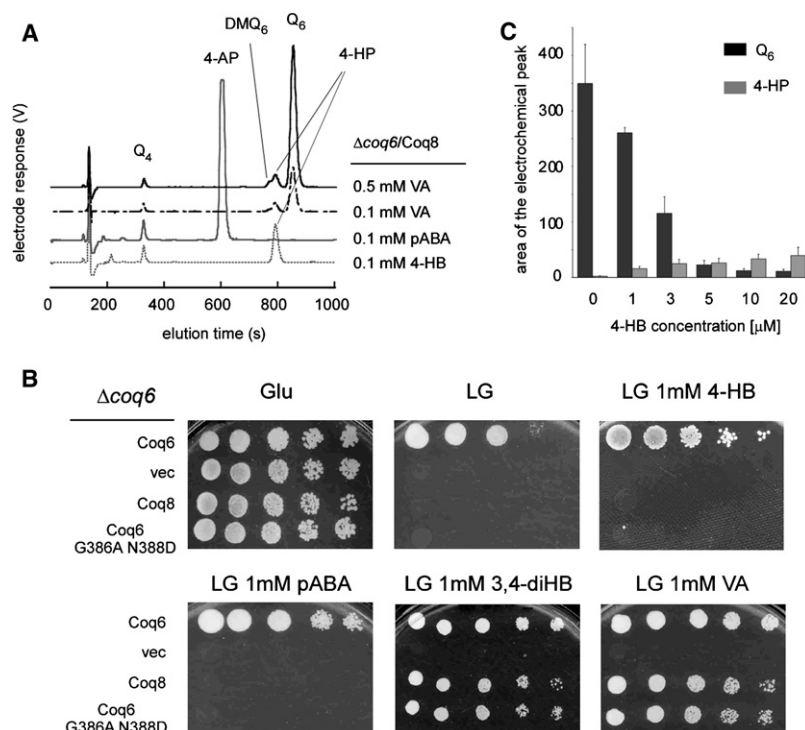


Figure 3. Vanillic Acid and 3,4-Dihydroxybenzoic Acid Restore Q₆ Biosynthesis and Respiration in *coq6* Mutant Cells

(A) $\Delta coq6$ cells transformed with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing the indicated concentrations of 4-HB, pABA, or vanillic acid (VA). Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(B) $\Delta coq6$ cells transformed either with an episomal vector coding for Coq8, an empty vector (vec) or with centromeric vectors coding for WT Coq6 or Coq6-G386A-N388D were grown in glucose synthetic medium for 24 hr and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented with 2% glucose (Glu) or 2% lactate-2% glycerol (LG) and either vanillic acid (VA), pABA, or 3,4-dihydroxybenzoic acid (3,4-diHB). The plates were incubated for 2 days (Glu) or 4 days (LG) at 30°C.

(C) Quantification of Q₆ and 4-HP formed in $\Delta coq6$ cells overexpressing Coq8 grown in glucose synthetic medium without pABA with 1mM VA and the indicated concentration of 4-HB. Error bars are standard deviation ($n = 2$). See also Figure S3.

strain containing only an empty vector synthesized no Q₆ from VA showing that the integrity of the Q biosynthetic complex is required to convert VA in Q₆ (Figure S3B). The $\Delta coq6$ strain overexpressing Coq8 or expressing Coq6-G386A-N388D was able to grow on respiratory carbon sources supplemented with 3,4-diHB or VA, showing that Q₆ biosynthesized from these substrate analogs is physiologically functional (Figure 3B). When pABA or 4-HB were added in place of VA or 3,4-diHB, the cells were not able to grow on respiratory substrates (Figure 3B), thus showing that 4-AP or 4-HP are not competent for electron transfer in the respiratory chain. VA also restored Q₆ biosynthesis in the $\Delta f/x1$ mutant to 70% of wild-type (WT) (Figure S3D).

Addition of increasing concentrations of 4-HB to the growth medium containing 1mM VA gradually decreased Q₆ levels and promoted the accumulation of 4-HP in the $\Delta coq6$ strain overexpressing Coq8 (Figure 3C). pABA had a similar impact on Q₆ levels at concentrations comparable to those of 4-HB (Figure S3E). Altogether our data show that exogenous pABA and 4-HB compete with VA to enter the Q₆ biosynthetic pathway at the prenylation step catalyzed by Coq2 and that prenylated VA is converted into Q₆, therefore bypassing the deficient C5-hydroxylation reaction in *coq6* mutant cells.

Coq6 and Yah1 Are Functionally Coupled in the C5-Hydroxylation Reaction

Our results show that the phenotypes of cells depleted for Yah1 (Pierrel et al., 2010) or deficient for Coq6 are strikingly similar with regard to Q₆ biosynthesis suggesting a mechanism in which Coq6 and Yah1 work together in the C5-hydroxylation step. The following experiments establish indeed that depletion of Yah1 directly impacts Coq6 activity. First, we demonstrate that

Coq6 is stable when Yah1 is depleted. We constructed a Gal-YAH1 COQ6-3HA strain that contains a chromosomal insertion of a sequence coding for a triple hemagglutinin (3HA) epitope tag on the 3' end of COQ6 and allows for regulated expression of Yah1. Indeed, the native YAH1 promoter has been replaced by the GAL1-10 promoter allowing for expression of Yah1 in a culture medium containing galactose and for depletion of Yah1 in a medium containing glucose (Lange et al., 2000). The Coq6-3HA protein was detected by immunoblotting at the expected size of 57 kDa (Figure 4A). Depletion of Yah1 by culturing the Gal-YAH1 COQ6-3HA strain in the presence of glucose had no effect on the steady-state level of Coq6 (Figure 4A). The presence of Coq6 in Yah1-depleted cells is also confirmed by the observation that VA or 3,4-diHB restores Q₆ biosynthesis in the Yah1-depleted cells regardless of Coq8 overexpression (Figure S4A), thus showing that the Q biosynthetic complex is intact and therefore that Coq6 is present. Second, overexpression of Fad1 did not complement the Q₆ biosynthetic defect in Yah1-depleted cells (Figure S4B), suggesting that this defect does not result from a depletion of mitochondrial FAD. These data together show that Yah1 depletion, although having a negative effect on Q₆ biosynthesis, has no effect on Coq6 stability or mitochondrial FAD content.

Human Fdx2 was recently shown to complement the ISC biosynthetic defect of Yah1-depleted cells (Sheftel et al., 2010). Surprisingly, none of the two human homologs of Yah1, Fdx1, or Fdx2 complemented the Q₆ defect of Yah1-depleted cells as shown from the observation that the cells contained almost no Q₆ and furthermore accumulated 4-AP upon culture in the presence of pABA (Figure 4B). Growth of the Gal-YAH1 strain on synthetic medium containing the respiratory carbon source lactate was limited unless it contained a plasmid carrying the YAH1 gene (Figure 4C). With a plasmid expressing Fdx2, growth was restored upon addition of VA or 3,4-diHB to the lactate medium whereas addition of 4-HB was without any effect

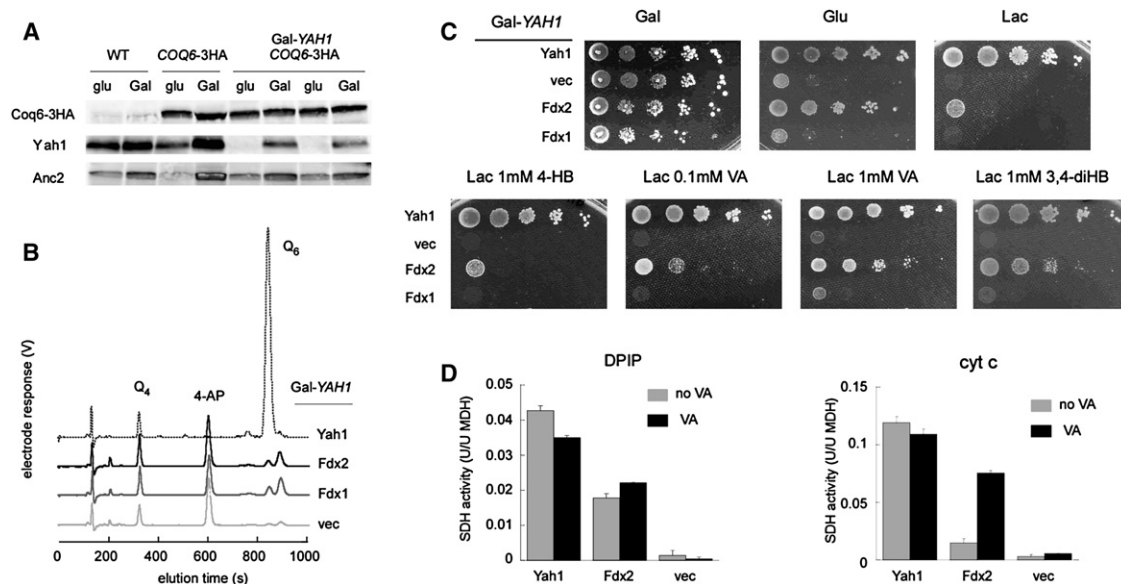


Figure 4. Q_6 Is Limiting for Respiratory Growth of Yah1-Depleted Cells Expressing Fdx2

(A) Immunoblot of whole-cell lysates from WT, COQ6-3HA, and Gal-YAH1 COQ6-3HA (two clones) cells grown in YP rich medium containing either 2% glucose (Glu) or 2% galactose (Gal). Coq6-3HA was detected at 57 kDa with an anti-HA antibody and as expected was absent from extracts of the WT strain. Yah1 was detected around 16 kDa and Anc2, the major ADP/ATP carrier of the mitochondrial inner membrane, at 35 kDa.

(B) Gal-YAH1 cells transformed with an empty vector (vec), a centromeric vector coding for Yah1 or episomal vectors coding for mitochondrially targeted human ferredoxin 1 or 2 (Fdx1 or Fdx2) were grown for 24 hr in synthetic medium supplemented with glucose and 1mM pABA. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(C) Same cells as in (B) were grown in glucose synthetic medium without pABA for 24 hr and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented at 2% with either glucose (Glu), galactose (Gal), or lactate (Lac) and either vanillic acid (VA), 3,4-dihydroxybenzoic acid (3,4-diHB), or 4-HB. The plates were incubated for 4 days at 30°C.

(D) Gal-YAH1 cells containing an empty vector (vec), a centromeric vector coding for Yah1 (Yah1) or an episomal vector coding for mitochondrially-targeted human ferredoxin 2 (Fdx2) were cultivated in glucose synthetic medium for 26 hr and finally in glucose synthetic medium without pABA in the presence or absence of 1 mM vanillic acid (VA) for 14 hr. Mitochondria were isolated and the enzyme activities of SDH (succinate to dichlorophenol-indophenol [DPIP]) and SDH in combination with complex III (succinate to cytochrome c [cyt c]) were determined and normalized to malate dehydrogenase activity (MDH). Error bars are standard deviation ($n = 3$).

See also Figure S4.

(Figure 4C). Our results thus suggest that, in the absence of VA or 3,4-diHB, Q_6 is limiting for respiratory growth of the Gal-YAH1 strain expressing Fdx2. This was confirmed by measuring the in vitro activity of succinate dehydrogenase (SDH), a multiprotein complex that contains three ISC and one FAD, all required for activity. As expected from the complementation of the ISC biosynthetic defect of Yah1-depleted cells by Fdx2 (Sheftel et al., 2010), Fdx2 significantly restored reduction of the chemical dichlorophenol-indophenol (DPIP) by SDH in Yah1-depleted cells, although not to WT levels (Figure 4D). Q_6 is required to transfer electrons from SDH to the cytochrome bc_1 complex that reduces cytochrome c (cyt c). Fdx2 restored SDH-cyt c reductase activity in Yah1-depleted cells only when VA was added to the culture medium (Figure 4D). This result shows that the limiting factor in transferring electrons from succinate to cyt c in Yah1-depleted cells expressing human Fdx2 is Q_6 , the level of which is restored by addition of VA to the growth medium (Figure S4A).

DISCUSSION

Our current work addresses the precise functional role of the Coq6 monooxygenase in the hydroxylation reactions of the Q

biosynthetic pathway. As shown previously in the case of the $\Delta coq7$ strain (Padilla et al., 2004, 2009), overexpression of Coq8 or expression of inactive alleles of COQ6 allow for preservation of the integrity of the Q_6 biosynthetic complex in a $\Delta coq6$ strain. We show here for the first time that under these conditions, the $\Delta coq6$ strain grown in the presence of pABA or 4-HB accumulates significant amounts of 4-AP and 4-HP, respectively. The accumulation of 4-AP and 4-HP is diagnostic of an impaired C5-hydroxylation but of a functional C1-hydroxylation. As a consequence, the predicted FAD-dependent monooxygenase Coq6 is required for the C5-hydroxylation but not for the C1-hydroxylation, definitively resolving the uncertainty regarding which hydroxylation reaction is catalyzed by Coq6. As a result, the C1-hydroxylase is still unknown (Figure 5) and is unlikely to be any of the Coq proteins identified to date because among these, only Coq6 and Coq7 display sequence homologies with monooxygenases and Coq7 participates exclusively in the C6-hydroxylation (Behan and Lippard, 2010; Padilla et al., 2004). Some experiments reported here suggest that the C1-hydroxylase is unlikely to be a FAD-dependent monooxygenase. A $\Delta flx1$ strain, characterized by impaired FAD-dependent mitochondrial activities (Bafunno et al., 2004; Tzagoloff et al., 1996), accumulates 4-AP and 4-HP indicating

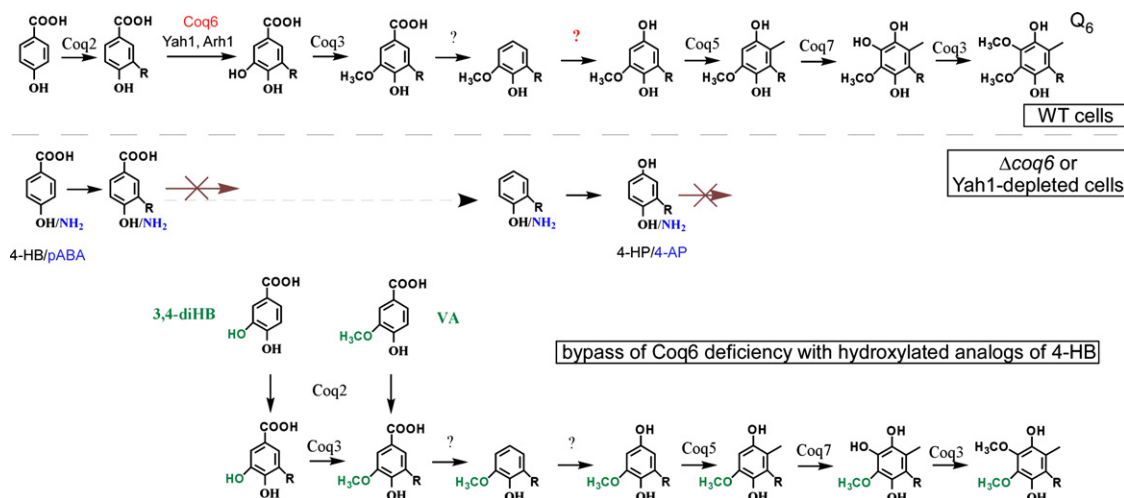


Figure 5. Coq6 Is Required for the C5-Hydroxylation of Q₆ Biosynthesis But Coq6 Deficiency Can Be Bypassed by Using Analogs of 4-HB

The pathway leading to Q₆ biosynthesis in WT *S. cerevisiae* cells is shown (above dashed line) with Coq6 implicated together with Yah1 and Arh1 in the C5-hydroxylation, whereas the C1-hydroxylation is catalyzed by an unidentified protein (?). In Yah1-depleted cells or in Δcoq6 cells overexpressing either COQ8 or an inactive COQ6 allele (below dashed line), the C5-hydroxylation does not take place (crossed arrow) but the C1-decarboxylation (dashed arrow) and the C1-hydroxylation proceed efficiently, leading to the accumulation of 4-HP when 4-HB is prenylated, or the accumulation of 4-AP when pABA is prenylated (blue). 3,4-diHB and VA contain an additional C5-hydroxyl (green) or C5-methoxyl (green) compared to 4-HB. 3,4-diHB and VA that correspond to the unprenylated products of the reactions catalyzed by Coq6 and Coq3 are prenylated in vivo by Coq2 and restore Q₆ biosynthesis in cells deficient for Coq6.

that the C1-hydroxylase is functional in this mutant (Figure 2C). Furthermore, restoration of Q₆ biosynthesis in Δ*flx1* strain by VA (Figure S3D) is consistent with the C1-hydroxylation proceeding normally in the Δ*flx1* strain.

Our results show that VA and 3,4-diHB can be used as Q₆ precursors and thus restore Q₆ biosynthesis and respiration in Δcoq6 cells overexpressing Coq8 or expressing the Coq6 inactive alleles (Figure 5). This proves that analogs of 4-HB added to the growth medium of *S. cerevisiae* can reach the mitochondrial matrix, where Q₆ biosynthesis takes place, and then enter the Q₆ biosynthetic pathway via prenylation by Coq2 (Figure 5). The strong impact of minor quantities of pABA or 4-HB observed on the levels of Q₆ synthesized from VA (Figure 3C and Figure S3E) may be explained by two nonexclusive hypotheses. VA may not be as efficiently transported to the mitochondrial matrix as pABA and 4-HB or Coq2 may have a higher affinity for pABA and 4-HB compared to VA. In any case, Coq2 has a broad substrate specificity in agreement with early in vitro studies that showed that mitochondrial preparations from rat heart and liver were able to prenylate VA and 3,4-diHB (Nambudiri et al., 1976). In fact, these analogs of 4-HB meet the structural requirements for prenylation by the polyprenyl transferase Coq2, i.e., an electron-donating substituent at position 4 of the aromatic ring combined with a carboxylic acid, a strong electron-attracting group, at position 1 (Alam et al., 1975). On the contrary, 4-nitrobenzoic acid that harbors the strong electron-attracting nitro group at position 4 inhibits Coq2 and as a consequence acts as an inhibitor of Q biosynthesis in mammalian cells cultures (Forsman et al., 2010). We would like to suggest that 4-HB analogs compatible with prenylation by Coq2 may also bypass deficient Q biosynthetic steps downstream of the C5-hydroxylation reaction. It is interesting to note that most mutations identified so far in humans to cause primary Q deficiency

are not found in genes encoding enzymes that catalytically modify the prenylated aromatic ring except for COQ6 (Heeringa et al., 2011). In fact they are found in (1) PDSS1 and PDSS2 (Lopez et al., 2006; Mollet et al., 2007), which are the relatives of COQ1 catalyzing the synthesis of the polyprenyl tail; (2) the polyprenyl transferase COQ2 (Mollet et al., 2007); (3) ADCK3/CABC1 (Lagier-Tourenne et al., 2008; Mollet et al., 2008), the COQ8 homolog; and (4) COQ9, a gene with no specific function assigned (Duncan et al., 2009). Our results suggest that VA may be efficient at promoting Q₁₀ biosynthesis in patients with COQ6 mutations. Practically, as vanillin (3-methoxy-4-hydroxybenzaldehyde), a common nontoxic food additive, is converted in the liver to VA (Muskiet and Groen, 1979; Sayavongsaa et al., 2007), vanillin may represent an interesting therapeutic molecule to try in patients with COQ6 mutations.

Finally, we previously demonstrated that Yah1 and Arh1 are essential for the C5-hydroxylation reaction (Pierrel et al., 2010). The similar impact on Q₆ biosynthesis of Yah1 or Arh1 depletion or Coq6 inactivation raises the question of how these three enzymes are functionally coupled. Here we provide preliminary data aiming at understanding this link. Yah1/Arh1 form a ferredoxin/ferredoxin reductase system that transfers electrons for different mitochondrial processes, in particular ISC assembly (Lill, 2009). The fact that Fdx2, a human homolog of Yah1, complements the ISC biosynthetic defect but not the Q₆ biosynthetic defect of Yah1-depleted cells excludes that Coq6 and thus Q₆ metabolism may indirectly be affected by an impairment of ISC biosynthesis. Coq6 is classified among Class A flavoprotein monooxygenases that have been described to contain a tightly bound FAD cofactor and to depend on NAD(P)H as a coenzyme for reduction of FAD (van Berkel et al., 2006). We checked whether inactivation of Yah1 could indirectly cause a depletion of mitochondrial NAD(P)H or FAD that would result in decreased

Coq6 activity. Our data summarized below indicate that, in the absence of Yah1, the Coq6 polypeptide is not degraded, mitochondrial FAD and NAD(P)H are available, and yet Coq6 is unable to perform the C5-hydroxylation, implying that Yah1 itself plays a role in this reaction. First, the Coq6-3HA polypeptide is detected in Yah1-depleted cells. Second, a shortage of mitochondrial FAD, the predicted cofactor of Coq6, is unlikely. Indeed, Yah1-depleted cells expressing Fdx2 are impaired for the C5-hydroxylation reaction but display significant SDH activity, known to depend on FAD. In addition, overexpression of *FAD1* restored Q₆ biosynthesis in the $\Delta f/x1$ strain whereas it failed to do so in Yah1-depleted cells. Third, mitochondrial NAD(P)H levels may not be dramatically affected in Yah1-depleted cells expressing Fdx2 because the latter is able to assemble ISC, a process supported by mitochondrial NADPH (Pain et al., 2010). Also in support of this, Yah1-depleted cells are prototroph for arginine (data not shown), the biosynthesis of which requires mitochondrial NADPH produced by Pos5 via phosphorylation of mitochondrial NADH (Outten and Culotta, 2003). As a conclusion, if Coq6 was a classical class A flavoprotein monooxygenase catalyzing reduction of FAD by NAD(P)H, it should be active in Yah1-depleted cells because their levels of mitochondrial FAD and NAD(P)H are not compromised. Consequently, we end up with the hypothesis of an unusual mechanism in which the reducing power of NAD(P)H may transit via the Yah1/Arh1 system before reaching Coq6. The β -cyclohexenyl carotenoid epoxidase, another class A flavoprotein monooxygenase, has been shown to require a ferredoxin/ferredoxin reductase system for in vitro activity (Bouvier et al., 1996) and thus represents a precedent for such an electron transfer pathway from a ferredoxin to a flavin-monooxygenase. Unequivocal demonstration that Coq6 may also use an unconventional reducing system like Arh1/Yah1 will necessitate the development of an in vitro assay with purified proteins; a challenging task given that Coq6 likely interacts with several Coq polypeptides in the Q₆ biosynthetic complex (Marbois et al., 2005). Nonetheless, our study has unambiguously established that Coq6 is required exclusively for the C5-hydroxylation of Q₆ biosynthesis and that hydroxylated analogs of 4-HB can be used as precursors of Q₆, two results that significantly improve our understanding of the biosynthesis of this crucial coenzyme.

SIGNIFICANCE

Coenzyme Q (Q) or ubiquinone, an important cellular antioxidant, is essential to electron transport chains and is required for several other cellular processes. Q biosynthesis requires at least 11 proteins in *S. cerevisiae* but the precise function of several of them is not known. Our work establishes that the predicted monooxygenase Coq6 is involved in the C5-hydroxylation reaction and that an unidentified monooxygenase catalyzes the C1-hydroxylation reaction. We further demonstrate the possibility to bypass a deficient Q biosynthetic step in *S. cerevisiae* by providing the defective chemical group within a synthetic 4-hydroxybenzoic acid analog. Indeed, a *coq6* mutant impaired in the C5-hydroxylation reaction recovers Q₆ biosynthesis and respiration on addition of two such analogs, 3,4-dihydroxybenzoic acid and vanillic acid. Primary Q₁₀ deficiencies have

been linked to mutations in six genes of the Q biosynthetic pathway and result in clinically heterogeneous diseases that, if diagnosed early, are improved by Q₁₀ supplementation. However, the lipophilicity of Q₁₀ may restrain its efficient transport to the mitochondrial inner membrane where Q₁₀ functions in the respiratory chain (Quinzii and Hirano, 2010). Our work suggests that hydrophilic analogs of 4-hydroxybenzoic acid may restore Q₁₀ biosynthesis in patients with some primary Q₁₀ deficiencies by bypassing the altered biosynthetic step. The use of various 4-hydroxybenzoic acid analogs will also contribute to characterize the biosynthetic step(s) blocked in some *S. cerevisiae* *coq* mutants and will thus help to identify the molecular function of Coq proteins with unknown function. Our work illustrates the importance of a molecular understanding of the Q biosynthetic pathway and warrants the identification of yet unidentified proteins that participates in Q biosynthesis, in particular the monooxygenase responsible for the C1-hydroxylation reaction.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions

S. cerevisiae strains used in this study are listed in Table S1. *S. cerevisiae* strains were transformed using lithium acetate. A 3HA epitope tag was inserted on the 3' end of COQ6 ORF by PCR as described previously (Longtine et al., 1998) to create the COQ6-3HA strain. This strain was crossed with the Gal-YAH1 strain to isolate the Gal-YAH1 COQ6-3HA strain by selecting the corresponding markers after tetrad dissection. YNB without pABA and folate was from MP Biomedicals. Rich YP medium was prepared as described (Sherman, 2002). Glucose, galactose, or lactate-glycerol was used at 2%. The Gal-YAH1 strain was maintained and precultured on galactose medium. Depletion of Yah1 was accomplished by diluting 200-fold the preculture into glucose containing medium and growing the cells for 18 hr at 30°C.

Plasmids

COQ6 ORF was cloned into pRS416 under the control of the MET25 promoter and the CYC1 terminator using XhoI and XbaI (Mumberg et al., 1994). This vector served as a template to generate the G202V and G386A-N388D Coq6 mutants by site directed mutagenesis. FAD1 was cloned with its own promoter (370 bp) and terminator (210 bp) into pRS423 using EcoRI and XhoI. Fdx1 and Fdx2 expressing plasmids have been described (Sheftel et al., 2010) and COQ8 cloned in pFL44 was a kind gift from Dr. Geneviève Dujardin. Sequencing was used to confirm cloning products in all created vectors.

Miscellaneous Biochemical Analysis

Isolation of mitochondria and immunostaining were performed as described (Diekert et al., 2001; Harlow and Lane, 1988). Cellular lipid extraction after addition of the Q₄ standard and quantification of electroactive compounds by HPLC-ECD with a 5011A analytical cell (E1, -420 mV; E2, +380 mV) were as described (Pierrel et al., 2010). Hydroquinones present in samples were oxidized with a precolumn 5020 guard cell set in oxidizing mode (E, +650 mV). Mitochondrial enzymatic activities were measured as previously described (Pierik et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.07.008.

ACKNOWLEDGMENTS

This work was supported in part by the Région Rhône-Alpes program CIBLE 2009 (to F.P.). U.M. and R.L. acknowledge generous support from Deutsche Forschungsgemeinschaft (SFB 593 and TR1), von Behring-Röntgen Stiftung,

LOEWE program of state Hessen, Max-Planck Gesellschaft, and Fonds der chemischen Industrie. We thank Dr. Sandrine Ollagnier and Dr. Gustav Berggren for critical reading of the manuscript.

Received: April 8, 2011

Revised: June 27, 2011

Accepted: July 6, 2011

Published: September 22, 2011

REFERENCES

- Alam, S.S., Nambudiri, A.M., and Rudney, H. (1975). J-Hydroxybenzoate: polyprenyl transferase and the prenylation of 4-aminobenzoate in mammalian tissues. *Arch. Biochem. Biophys.* 171, 183–190.
- Bafunno, V., Giancaspero, T.A., Brizio, C., Bufano, D., Passarella, S., Boles, E., and Barile, M. (2004). Riboflavin uptake and FAD synthesis in *Saccharomyces cerevisiae* mitochondria: involvement of the Flx1p carrier in FAD export. *J. Biol. Chem.* 279, 95–102.
- Barros, M.H., Nobrega, F.G., and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277, 9997–10002.
- Behan, R.K., and Lippard, S.J. (2010). The aging-associated enzyme CLK-1 is a member of the carboxylate-bridged diiron family of proteins. *Biochemistry* 49, 9679–9681.
- Bentinger, M., Tekle, M., and Dallner, G. (2010). Coenzyme Q—biosynthesis and functions. *Biochem. Biophys. Res. Commun.* 396, 74–79.
- Bouvier, F., d'Harlingue, A., Huguency, P., Marin, E., Marion-Poll, A., and Camara, B. (1996). Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J. Biol. Chem.* 271, 28861–28867.
- Diekert, K., de Kroon, A.I., Kispal, G., and Lill, R. (2001). Isolation and subfractionation of mitochondria from the yeast *Saccharomyces cerevisiae*. *Methods Cell Biol.* 65, 37–51.
- Duncan, A.J., Bitner-Glindzicz, M., Meunier, B., Costello, H., Hargreaves, I.P., López, L.C., Hirano, M., Quinzii, C.M., Sadowski, M.I., Hardy, J., et al. (2009). A nonsense mutation in COQ9 causes autosomal-recessive neonatal-onset primary coenzyme Q10 deficiency: a potentially treatable form of mitochondrial disease. *Am. J. Hum. Genet.* 84, 558–566.
- Eppink, M.H., Schreuder, H.A., and Van Berkel, W.J. (1997). Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding. *Protein Sci.* 6, 2454–2458.
- Forsman, U., Sjöberg, M., Turunen, M., and Sindelar, P.J. (2010). 4-Nitrobenzoate inhibits coenzyme Q biosynthesis in mammalian cell cultures. *Nat. Chem. Biol.* 6, 515–517.
- Gin, P., Hsu, A.Y., Rothman, S.C., Jonassen, T., Lee, P.T., Tzagoloff, A., and Clarke, C.F. (2003). The *Saccharomyces cerevisiae* COQ6 gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. *J. Biol. Chem.* 278, 25308–25316.
- Harlow, E., and Lane, D. (1988) (Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press).
- Heeringa, S.F., Chernin, G., Chaki, M., Zhou, W., Sloan, A.J., Ji, Z., Xie, L.X., Salviati, L., Hurd, T.W., Vega-Warner, V., et al. (2011). COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness. *J. Clin. Invest.* 121, 2013–2024.
- Hsieh, E.J., Gin, P., Gulmezian, M., Tran, U.C., Saiki, R., Marbois, B.N., and Clarke, C.F. (2007). *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch. Biochem. Biophys.* 463, 19–26.
- Kawamukai, M. (2009). Biosynthesis and bioproduction of coenzyme Q10 by yeasts and other organisms. *Biotechnol. Appl. Biochem.* 53, 217–226.
- Lagier-Tourenne, C., Tazir, M., Lopez, L.C., Quinzii, C.M., Assoum, M., Drouot, N., Busso, C., Makri, S., Ali-Pacha, L., Benhassine, T., et al. (2008). ADCK3, an ancestral kinase, is mutated in a form of recessive ataxia associated with coenzyme Q(10) deficiency. *Am. J. Hum. Genet.* 82, 661–672.
- Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000). A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. *Proc. Natl. Acad. Sci. USA* 97, 1050–1055.
- Li, J., Saxena, S., Pain, D., and Dancis, A. (2001). Adrenodoxin reductase homolog (Arl1p) of yeast mitochondria required for iron homeostasis. *J. Biol. Chem.* 276, 1503–1509.
- Lill, R. (2009). Function and biogenesis of iron-sulphur proteins. *Nature* 460, 831–838.
- Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Lopez, L.C., Schuelke, M., Quinzii, C.M., Kanki, T., Rodenburg, R.J., Naini, A., Dimauro, S., and Hirano, M. (2006). Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyl diphosphate synthase subunit 2 (PDSS2) mutations. *Am. J. Hum. Genet.* 79, 1125–1129.
- Marbois, B., Gin, P., Faull, K.F., Poon, W.W., Lee, P.T., Strahan, J., Shepherd, J.N., and Clarke, C.F. (2005). Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. *J. Biol. Chem.* 280, 20231–20238.
- Marbois, B., Xie, L.X., Choi, S., Hirano, K., Hyman, K., and Clarke, C.F. (2010). para-Aminobenzoic acid is a precursor in coenzyme Q6 biosynthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 285, 27827–27838.
- Mollet, J., Giurgea, I., Schlemmer, D., Dallner, G., Chretien, D., Delahodde, A., Bacq, D., de Lonlay, P., Munnich, A., and Rötig, A. (2007). Prenyldiphosphate synthase, subunit 1 (PDSS1) and OH-benzoate polyprenyltransferase (COQ2) mutations in ubiquinone deficiency and oxidative phosphorylation disorders. *J. Clin. Invest.* 117, 765–772.
- Mollet, J., Delahodde, A., Serre, V., Chretien, D., Schlemmer, D., Lombes, A., Boddaert, N., Desguerre, I., de Lonlay, P., de Baulny, H.O., et al. (2008). CABC1 gene mutations cause ubiquinone deficiency with cerebellar ataxia and seizures. *Am. J. Hum. Genet.* 82, 623–630.
- Mumberg, D., Müller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22, 5767–5768.
- Muskiet, F.A.J., and Groen, A. (1979). Urinary excretion of conjugated homovanillic acid, 3,4-dihydroxyphenylacetic acid, p-hydroxyphenylacetic acid, and vanillic acid by persons on their usual diet and patients with neuroblastoma. *Clin. Chem.* 25, 1281–1284.
- Nambudiri, A.M.D., Brockman, D., Alam, S.S., and Rudney, H. (1976). Alternate routes for ubiquinone biosynthesis in rats. *Biochem. Biophys. Res. Commun.* 76, 282–288.
- Outten, C.E., and Culotta, V.C. (2003). A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. *EMBO J.* 22, 2015–2024.
- Padilla, S., Jonassen, T., Jiménez-Hidalgo, M.A., Fernández-Ayala, D.J., López-Lluch, G., Marbois, B., Navas, P., Clarke, C.F., and Santos-Ocaña, C. (2004). Demethoxy-Q, an intermediate of coenzyme Q biosynthesis, fails to support respiration in *Saccharomyces cerevisiae* and lacks antioxidant activity. *J. Biol. Chem.* 279, 25995–26004.
- Padilla, S., Tran, U.C., Jiménez-Hidalgo, M., López-Martín, J.M., Martín-Montalvo, A., Clarke, C.F., Navas, P., and Santos-Ocaña, C. (2009). Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis. *Cell. Mol. Life Sci.* 66, 173–186.
- Pain, J., Balamurali, M.M., Dancis, A., and Pain, D. (2010). Mitochondrial NADH kinase, Pos5p, is required for efficient iron-sulfur cluster biogenesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 285, 39409–39424.
- Palfey, B.A., Entsch, B., Ballou, D.P., and Massey, V. (1994). Changes in the catalytic properties of p-hydroxybenzoate hydroxylase caused by the mutation Asn300Asp. *Biochemistry* 33, 1545–1554.
- Pierik, A.J., Netz, D.J., and Lill, R. (2009). Analysis of iron-sulfur protein maturation in eukaryotes. *Nat. Protoc.* 4, 753–766.
- Pierrel, F., Hamelin, O., Douki, T., Kieffer-Jaquinod, S., Mühlhoff, U., Ozeir, M., Lill, R., and Fontecave, M. (2010). Involvement of mitochondrial ferredoxin

- and para-aminobenzoic acid in yeast coenzyme Q biosynthesis. *Chem. Biol.* **17**, 449–459.
- Quinzii, C.M., and Hirano, M. (2010). Coenzyme Q and mitochondrial disease. *Dev. Disabil. Res. Rev.* **16**, 183–188.
- Sayavongsaa, P., Cooperb, M.L., Jacksona, E.M., Harrisa, L., Zieglerc, T.R., and Hibbert, J.M. (2007). Vanillic acid excretion can be used to assess compliance with dietary supplements. *e-SPEN* **2**, e134–e137.
- Sheftel, A.D., Stehling, O., Pierik, A.J., Elsässer, H.P., Mühlenhoff, U., Webert, H., Hobler, A., Hannemann, F., Bernhardt, R., and Lill, R. (2010). Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2, with distinct roles in steroidogenesis, heme, and Fe/S cluster biosynthesis. *Proc. Natl. Acad. Sci. USA* **107**, 11775–11780.
- Sherman, F. (2002). Getting started with yeast. In *Guide to Yeast Genetics and Molecular and Cell Biology*, Pt B, *Volume 350*, C. Guthrie and G.R. Fink, eds. (San Diego: Academic Press), pp. 3–41.
- Stenmark, P., Grünler, J., Mattsson, J., Sindelar, P.J., Nordlund, P., and Berthold, D.A. (2001). A new member of the family of di-iron carboxylate proteins. Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis. *J. Biol. Chem.* **276**, 33297–33300.
- Tauche, A., Krause-Buchholz, U., and Rodel, G. (2008). Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of O-methylase Coq3p depends on Abc1p/Coq8p. *FEMS Yeast Res.* **8**, 1263–1275.
- Tran, U.C., and Clarke, C.F. (2007). Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion Suppl.* **7**, S62–S71.
- Tzagoloff, A., Jang, J., Glerum, D.M., and Wu, M. (1996). FLX1 codes for a carrier protein involved in maintaining a proper balance of flavin nucleotides in yeast mitochondria. *J. Biol. Chem.* **271**, 7392–7397.
- van Berkel, W.J.H., Kamerbeek, N.M., and Fraaije, M.W. (2006). Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J. Biotechnol.* **124**, 670–689.
- Wu, M., Repetto, B., Glerum, D.M., and Tzagoloff, A. (1995). Cloning and characterization of FAD1, the structural gene for flavin adenine dinucleotide synthetase of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**, 264–271.
- Xie, L.X., Hsieh, E.J., Watanabe, S., Allan, C.M., Chen, J.Y., Tran, U.C., and Clarke, C.F. (2011). Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast coq8 mutants. *Biochim. Biophys. Acta* **1811**, 348–360.
- Zampol, M.A., Busso, C., Gomes, F., Ferreira-Junior, J.R., Tzagoloff, A., and Barros, M.H. (2010). Over-expression of COQ10 in *Saccharomyces cerevisiae* inhibits mitochondrial respiration. *Biochem. Biophys. Res. Commun.* **402**, 82–87.